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PRINCIPAL INVESTIGATOR: Alexey V. Terskikh, Ph.D.

CONTRACTING ORGANIZATION: Burnham Institute for Medical Research
La Jolla, CA 92037

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Alexey V. Terskikh, Ph.D.

E-Mail: terskikh@burnham.org

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14. ABSTRACT

We have performed 3 rounds of in silico docking combined with in vitro MELK kinase activity assays. We have identified 5 independent scaffolds capable of MELK inhibition in the range of 100nm-1um. These compounds are capable to tightly bind MELK catalytic domain as measured by DCS, ruling out the protein aggregation and other non-specific inhibiotry modalities. These leads are now being developed into potent anti-MELK drugs.

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Introduction

Maternal Leucine Zipper Kinase (MELK) was found to be overexpressed in undifferentiated breast cancers. In a transgenic mouse model, MELK expression labels endogenous mammary tumors, the mammary gland end buds, and proliferating neural precursors in the brain (Nakano et al., 2005; Rhodes et al., 2002). Inhibition of MELK function is deleterious for several types of tumors, including human breast cancer and experimental mammary tumors (Gray et al., 2005). Among all human kinases, MELK has a unique amino acid sequence in the catalytic loop of the kinase domain. Computer modeling of the MELK catalytic loop revealed that several unusual cysteine residues reside in the ATP-binding pocket. Previously published data support the requirement of free cysteines for MELK activity (Beullens et al., 2005). The availability of free cysteines was used successfully to create irreversible covalent inhibitors of RSK kinases (Cohen et al., 2005). Very recently, we used molecular docking to design specific covalent inhibitors of beta-catenin and non-steroidal antagonists of the androgen receptor (submitted).

We propose that irreversible covalent inhibitors of MELK kinase will efficiently destroy mammary tumors in vitro and in vivo in animal models and allow further the development of therapeutic drugs to treat breast cancer in a clinical setting.

We will identify initial compounds by in silico docking into the MELK catalytic loop, synthesize and assay candidates for inhibitory activity using surrogate and physiological targets of MELK. We will develop second/third generations of covalent MELK inhibitors (IC50 0.1-1μM) using iterative rounds of in silico docking and synthesis/assay of compounds and physiological targets of MELK.

Body

We used a variation of a ligand-assisted protein structure optimization protocol. Briefly, ATP molecule was docked into the original MELK model . Several ATP binding modes were selected and BPMC was applied to the protein side chains in the vicinity of the molecule, creating a set of ATP binding pocket conformations. Then, the panel of known inhibitors of kinases homologous to MELK was compiled (50 compounds) and docked into previously generated MELK conformers. The top binders were assayed in vitro, and ligands exhibiting significant MELK

Optimization Round	Ligand(s)	IC_{50}, μM
I	U0126	8.5 ± 0.5
	HA-1004	11.0 ± 1.0
	HA-100	--+
Source: known inhibitors	Roscovitin	--+
II	H89	4.9 ± 0.2
	TBB	---
	SB203580	---
Source: known inhibitors	SL237	---
III	NCI4	0.9 ± 0.1
	NCI8	0.20 ± 0.07
	ENM10	0.5 ± 0.1
	CBR1	0.16 ± 0.04
Source: commercial libraries	CMD6	2.3 ± 0.2

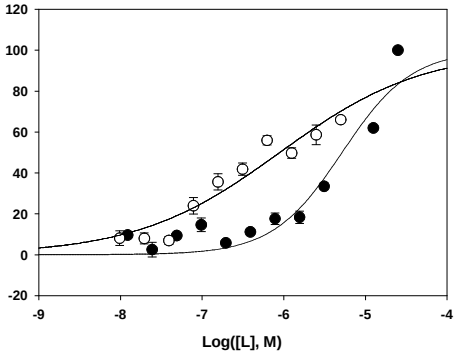

FIGURE 1. **Iterative optimization of MELK inhibitors. Left.** Optimization rounds. In those cases where IC_{50} values are not available, inhibitory potency is indicated as [---] for insignificant inhibition, and [+++] for significant inhibition. For Round III, the data for only top inhibitors from a total of 50 compounds are shown. **Right.** Examples of IC_{50} curves. Data were fit to variable slope dose response equation. 10 μM ATP; 37C; 1 hour incubation; 40 mM TRIS-HCl, pH 7.5; 20 mM $MgCl_2$; 150 mM NaCl; 0.1 mg/ml BSA; 20 μg/ml MBP; 1 μg/ml MELK.

inhibition (IC50< 1 mM) were selected for docking and further conformers refinement. This step in the protocol implicitly models induced fit of the protein in response to ligand binding. The protocol was applied iteratively in three rounds . The compounds in rounds I and II were selected from a panel of known kinase inhibitors, while the compound library for round III was built by searching through available commercial libraries. The search was based on Tanimoto distance from the best binders identified at rounds I and II.

The Round III compound library contained approximately 10,000 compounds. From those, 50 were purchased and screened in vitro. The examples of IC50 curves for different ligands are shown on figure 1.The specificity of inhibition of certain ligands was also validated by DSC technique.

Differential scanning calorimetry (DSC) is a technique able to study thermally induced transitions, in particular, the conformational transitions of biological macromolecules. When a macromolecule changes its thermodynamic state (e.g., unfolds), a heat capacity change (ΔCp) is observed. This change is due to the fact that the heat required to raise the temperature of a solution of unfolded protein is greater than that required for a solution of folded protein. The observed excess heat results primarily from restructuring of the solvent molecules around the side chains of the hydrophobic residues, exposed during the protein unfolding. DSC measures the

excess heat capacity of a solution (Cp) of a protein as a function of a temperature. The position

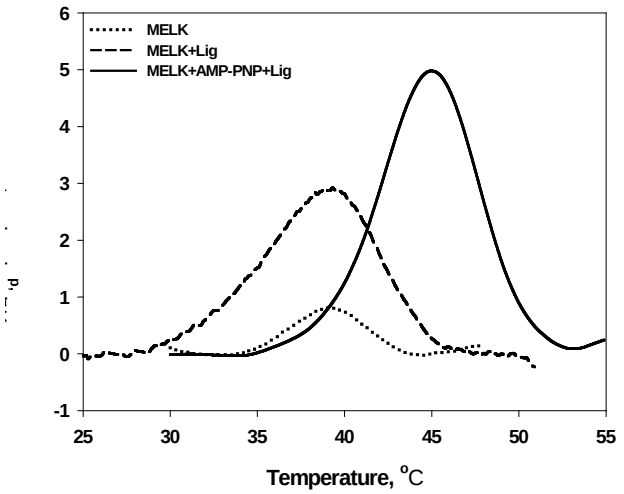

FIGURE 4. **Differential scanning calorimetry experiments.** MELK catalytic domain in complex with pilot inhibitor (T_m=39.3 ^{0}C; ΔH=23.0 kcal/mol) and with both AMP-PNP and a pilot inhibitor /Lig/ (T_m=45.3 ^{0}C; ΔH=32.3 kcal/mol).

of the transition peak corresponds to the protein melting temperature (Tm), while the area under the peak correlates with the content of ordered secondary structure of a protein . The main advantages of DSC are that it is not affected by the optical properties of the sample (as opposed to CD and fluorescence measurements), and that DSC experiments can be performed on native (unlabeled) proteins. DSC was successfully used before to characterize kinases structural state and how it affected by ligands.

Purified MELK catalytic domain was further characterized by DSC to assess the folding state of the recombinant protein (Fig. 2). The melting temperature of the MELK was found to be 38 0C with rather small area under the transition peak, which altogether suggests poor folding of the recombinant protein. However, in the presence of either non-hydrolysable ATP analog or our pilot inhibitor, significant stabilization of the protein's tertiary structure was observed, as suggested by transition peak area increase. Moreover, when both non-hydrolysable ATP analog and pilot compound were added to the protein, 6 degrees increase of melting temperature was achieved.

Task 2. Develop a second/third generation of compounds based on the initial leads using iterative
rounds of in silico docking and assay/synthesis of compounds. (Months 6-10):

Key Research Accomplishments

Task 1. Identify initial set of compounds through in silico docking and assay MELK activity using surrogate and native phosphorylation targets.

In order to perform Task 1, we have: First, generated and characterized MELK catalytic domain and NusA-MELK and GST-MELK fusion proteins, critical for developing MELK inhibitors; Second: screened ~50 compound and generated 5 independent MELK inhibitors with the IC50 ranging from 1μm to 100nm. We have did not proceed to the screening of another 50 comounds because of the virtual docking space saturation making it unnecessary continueing the primary screening. Instead, we focused our efforts on the second round of screening.

Task 2. Develop a second/third generation of compounds based on the initial leads using iterative rounds of in silico docking and assay/synthesis of compounds.

a. based on the results the refined/modified structures of active compound followed by another round of in silico docking. (Months 6-7).

b. repeat step in Task 2a to obtain a panel of diverse inhibitors with IC50 around 1-10uM

We have perfomed 2 rounds of screening and generated the compounds in the 2nd and 3d rounds. We generated 4 comounds with IC50 below the projected IC50 (1μM), in particular one compound with the IC50 0.16μM, which is ~7 times better than projected.

Task 3 was not performed as planned due to the unforeseen low stability of MELK catalytic domain and thus the necessity to first characterize the binding of obtained compounds using the advanced protein stability assay (DSC).

We demonstrated that MELK inhibitors inhibit the catalytic function of MELK kinase and showed that this inhibition is due to a tight binding / protein stabilization and not protein aggregation (the DCS data).

Reportable Outcomes

We have obtained 5 MELK inhibitors, 4 of them with the IC50 below 1 μM.

The list of obtained inhibitors: NCI4, NCI8, CDM6 CDR1 ENM10.

This work was the basis for an R21 application that has been currently reviewed.

The manuscript is currently being prepared. This funded supported a salary of a talented postdoctoral fellow Anton Cheltsov, PhD.

Conclusion

The completed research lays a foundation for generation of lead compounds inhibiting the MELK catalytic function. We have made a seminal discovery generating a set of unique small molecule scaffolds which will be further refined in my laboratory to generate the small molecule inhibitors of MELK catalytic function useful for research and clinical application.

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